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A Phytochrome Sensory Domain Permits Receptor Activation by Red Light

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Abstract: *Optogenetics and photopharmacology enable the spatio-temporal control of cell and animal behavior by light. Although red light offers deep-tissue penetration and minimal phototoxicity, very few red-light-sensitive optogenetic methods are currently available. We have now developed a red-light-induced homodimerization domain. We first showed that an optimized sensory domain of the cyanobacterial phytochrome 1 can be expressed robustly and without cytotoxicity in human cells. We then applied this domain to induce the dimerization of two receptor tyrosine kinases—the fibroblast growth factor receptor 1 and the neurotrophin receptor trkB. This new optogenetic method was then used to activate the MAPK/ERK pathway non-invasively in mammalian tissue and in multicolor cell-signaling experiments. The light-controlled dimerizer and red-light-activated receptor tyrosine kinases will prove useful to regulate a variety of cellular processes with light.*

Optogenetics has revolutionized neuroscience and cell biology by providing optical, thus spatially and temporally precise, means to decode molecular and cellular networks. The regulation of protein function by light is often achieved through genetically engineered intra- or intermolecular binding reactions.[1] For example, in the light-activated GTPase Rac1,[2] a C-terminal helix dissociates from its light-oxygen-voltage sensing (LOV) domain core to result in inhibition of enzyme function. In light-activated gene regulation and membrane recruitment, LOV domains either homodimerize or cryptochromes heterodimerize with interacting factors in functionally relevant complexes.[3] In these photoreceptors, flavin cofactors (flavin mononucleotide or flavin adenine dinucleotide) endow sensitivity to blue light (Figure 1 a). However, blue light overlaps with wavelengths used for the imaging of many fluorophores and exhibits limited tissue penetration. The application of red light can overcome these limitations, but is restricted to reactions of cyclic (di)nucleotides or heterodimerization.[4] Here, we extended the arsenal of light-controlled reactions with a red-light-sensing homodimerizer, which we repurposed from the cyanobacterial phytochrome 1 (CPH1) of *Synechocystis* and applied to activate membrane receptors orthogonally from fluorescent proteins and in mammalian tissue.

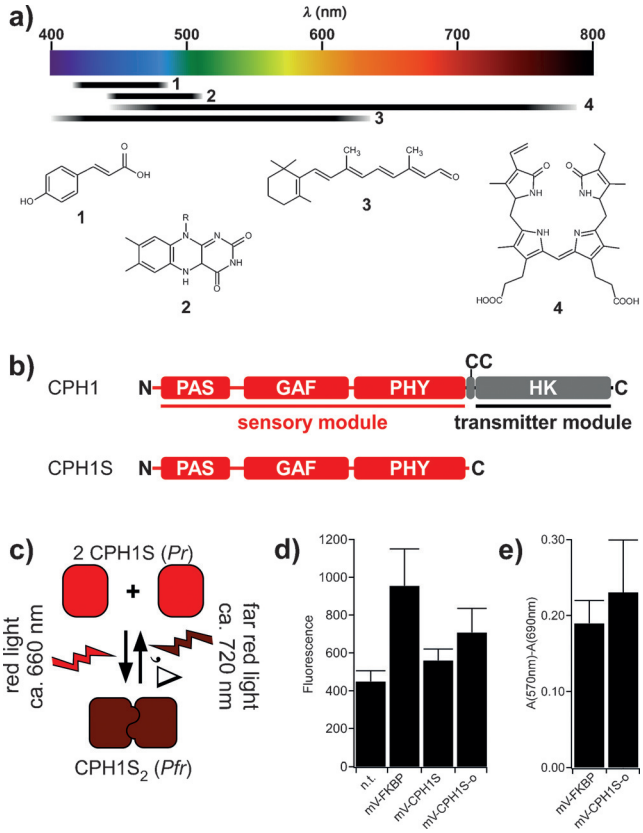

Figure 1. a) Chromophores of main photoreceptor classes (**1**: *p*-coumaric acid, **2**: flavins, **3**: retinal, **4**: tetrapyrroles). b) Domain structure of CPH1 with light sensory (CPH1S) and transmitter modules (PAS: Per Arnt Sim; GAF: cGMP-specific phosphodiesterase, adenylyl cyclase, and FhlA; PHY: phytochrome; HK: histidine kinase). c) CPH1S can be interconverted between a mainly monomeric Pr state and mainly homodimeric Pfr state. d) Fluorescence intensity of HEK293 cells expressing CPH1S, CPH1S-o, and FKBP tagged with mVenus (mV). n.t.: nontransfected cells. e) Viability of HEK293 cells transfected with mV-FKBP or mV-CPH1S-o. In (d) and (e), mean values ± standard deviation are shown for three independent experiments each performed in triplicate.

Phytochromes form a diverse protein family in microbes and plants that senses light through bound linear tetrapyrroles (Figure 1 a).[5] In particular, some phytochromes, including CPH1, are converted between red-light-absorbing Pr states and far-red-light-absorbing Pfr states. It was shown by in vitro experiments that the sensory module of CPH1 (CPH1S; Figure 1 b), which corresponds to the C-terminally truncated

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protein, undergoes light-dependent changes in the oligomerization state.[6] Red light resulted in the homodimeric Pfr state and far-red light in the monomeric Pr state (Figure 1c). We hypothesized that this domain may be used to control protein–protein interactions in mammalian cells through the use of red light. As CPH1S was not expressed in mammalian cells previously, we tested if its biogenesis is harmonious with the production of mammalian protein in human embryonic kidney 293 (HEK293) cells. We first fused a 1539 base pair fragment (CPH1S) from genomic DNA to the bright yellow fluorescent protein mVenus, which allowed for quantification of CPH1S expression in the context of a fusion protein. We observed weak fluorescence in HEK293 cells transfected with the mVenus–CPH1S construct (Figure 1d), which was indicative of poor protein expression, which may impede its straightforward use. We reasoned that limited expression might originate from divergent codon usage of the cyanobacterial gene and mammalian host cells.[7] Indeed, a synthetic codon-optimized variant of the gene (CPH1S-o) showed increased expression without measurable cytotoxicity (Figure 1d,e).

Homodimerization underlies the function of diverse protein families, such as kinases, cadherins, antibodies, motor proteins, and transcription factors. For example, it was shown previously for many receptor tyrosine kinases (RTKs) that homodimerization is required and can be sufficient for the initiation of downstream signaling pathways, such as mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) or phosphatidylinositol-3 kinase/Akt (PI3K/Akt) pathways.[8] To create a red-light-activated RTK, we fused CPH1S-o to the far C terminus of the murine fibroblast growth factor receptor 1 (mFGFR1; Figure 2a). Chemical homodimerization domains, such as an engineered FK506 binding protein (FKBP), or fluorescent proteins, were previously incorporated at this position.[9] The extracellular domain of the receptor was replaced by a myristoylation domain to obtain a receptor that is inert to its natural ligands.[9a,10] We next examined the ability of mFGFR1–CPH1S-o to activate the MAPK/ERK pathway in HEK293 cells in response to red light. Cells supplemented with the tetrapyrrole phycocyanobillin (PCB) responded to dim red light ($I = 6.2\ \mu W\,cm^{-2}$, $\lambda \approx (630 \pm 5)$ nm) with a strong pathway activation, as measured using a transcriptional reporter (Figure 2b). Control experiments showed that 1) blue ($I = 150\ \mu W\,cm^{-2}$, $\lambda \approx (470 \pm 5)$ nm) or green ($I = 6.2\ \mu W\,cm^{-2}$, $\lambda \approx (530 \pm 5)$ nm) light did not result in pathway activation, 2) red light had no effect after loss of the kinase activity (Y271F and Y272F mutations) or on a fusion protein of mFGFR1 and FKBP (Figure 2b). Notably, the results obtained with mFGFR1–FKBP indicated that mFGFR1–CPH1S-o did not dimerize in the dark. Finally, substituting a conserved arginine (R195) in the mFGFR1 catalytic domain with a glutamate, which prevents formation of an essential, asymmetric kinase domain dimer,[11] abolished pathway activation, thus indicating that dimerization is required for activation of the receptor by CPH1S-o (Figure 2b).

We extended this design principle to a second RTK, the rat neurotrophin receptor trkB (rtrkB). In this case, red light also resulted in a pathway activation (Figure 2c) that depended on PCB (see Figure S1 in the Supporting Information). Immunoblotting demonstrated that activation of the MAPK/ERK and PI3K/Akt pathways returned to basal levels about 30 min after illumination with red light (Figure S2).

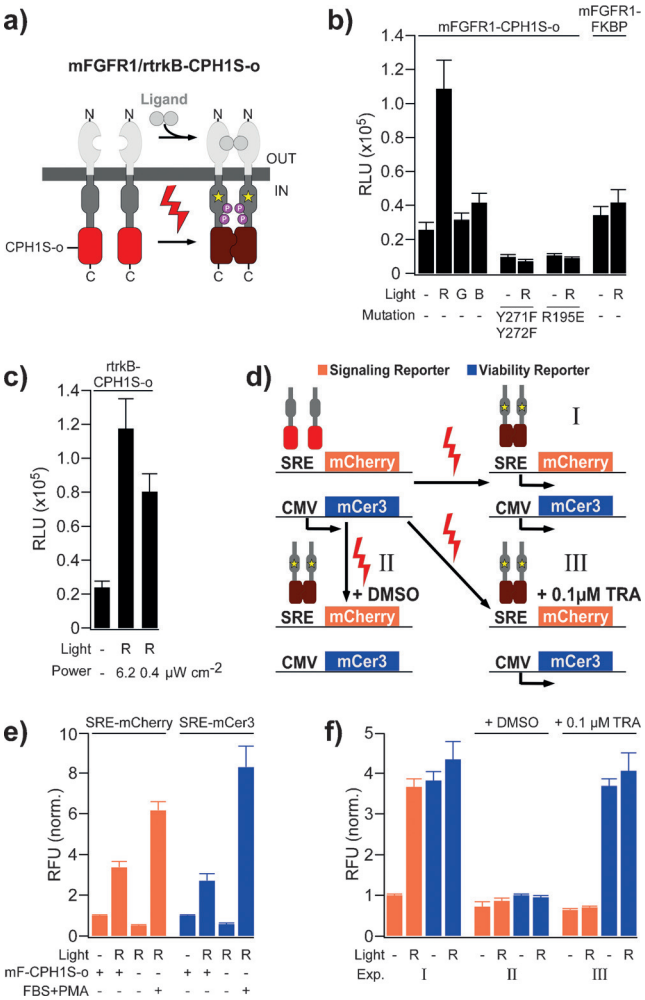

Figure 2. a) CPH1S-o (red) was incorporated in mFGFR1 or rtrkB to engineer red-light-activated receptors. b),c) activation of the MAPK/ERK pathway by mFGFR1–CPH1S-o (b) and rtrkB–CPH1S-o (c) in response to red (R), green (G), and blue (B) light. Pathway activation is expressed as reporter gene levels in HEK293 cells. d) Multicolor experiments to test receptor/pathway inhibition and cell viability. I) CMV–mCerulean3 is expressed constitutively, whereas SRE–mCherry depends on activation of the MAPK/ERK pathway (mCer3: mCerulean3). II) The addition of DMSO leads to cell death, thereby resulting in expression of neither mCherry nor mCerulean3. III) The addition of 0.1 µM Trametinib (TRA) prevents activation of the MAPK/ERK pathway, whereas viability is not affected. e) Activation of the MAPK/ERK pathway by mFGFR1–CPH1S-o in response to red light measured with SRE–mCherry/–mCerulean3. FBS/PMA was used as a positive control. Activation is expressed in terms of the mCherry or mCerulean3 levels. f) Experimental realization of (d). Activation of the MAPK/ERK pathway in response to red light, after treatment of the cells with DMSO or TRA. Pathway activation and cell viability is expressed as mCherry and mCerulean3 levels, respectively. In (b) and (c), mean values ± standard errors of the mean for 2–17 independent experiments, each performed in triplicate, are shown. In (e) and (f), mean values ± standard errors of the mean for three independent experiments, each performed in triplicate, are shown.

Overall, incorporation of CPH1S-o, but not of other cyano- or proteobacterial phytochromes (Figure S3), into RTKs enabled activation of the receptors and key pathways by red light.

Light absorbed by CPH1 and other phytochromes is red-shifted compared to that absorbed by many fluorescent proteins (e.g. typical blue, green, and red fluorescent proteins are excited at $\lambda \approx 440$, 490, and 560 nm, respectively). This property has been used to an advantage in studies that applied phytochromes as spectrally separated fluorophores in multicolor imaging.[12] Inspired by this work, we explored whether CPH1S-o can be combined with fluorescent proteins (e.g. mCerulean3: $\lambda(E_x/E_m) = 433/475$ nm, mCherry: $\lambda(E_x/E_m) = 587/610$ nm).[13] The goal of these experiments was optical actuation of cells (CPHS1-o), combined with optical detection of their signaling state (the first fluorescent protein) and viability (the second fluorescent protein; Figure 2d). We first engineered transcriptional fluorescent reporters of the MAPK/ERK pathway in which mCerulean3 or mCherry were under control of the pathway-sensitive serum response element (SRE).[14] We next tested whether these proteins are indeed spectrally separated from the red light used for the activation of CPH1S-o. As expected, we found that red light did not result in the bleaching of these two proteins (Figure S4), and thus the reporters can faithfully detect pathway activation by mFGFR1–CPH1S-o (Figure 2e). We then used the three components to demonstrate that light-induced cell signaling can be detected separately from cell viability in the same experiment. We prepared a viability reporter in which mCerulean3 was under the control of the constitutively active cytomegalovirus (CMV) promoter, and then applied this viability reporter in combination with the mCherry MAPK/ERK pathway reporter. We incubated cells either in a high concentration of DMSO to induce cell death or in the specific MAPK/ERK pathway inhibitor Trametinib (TRA; Figure 2d). The addition of TRA reduced the red-light-induced mCherry signal but not the mCerulean3 signal (Figure 2f). In contrast, the addition of DMSO led to the reduction of both signals (Figure 2f). Thus, taking advantage of the spectral separation of CPH1S-o, we showed that effects of specific inhibitors can be distinguished from those of unspecific toxins in a single all-optical experiment. In future experiments, it may be possible to use different colors of light to independently activate two or more processes in the same cell or two or more cell types in a heterogeneous population to study cross-talk of cellular signaling pathways or cell–cell interactions.

An important trait of red light is its ability to penetrate tissue more deeply than blue or green light. Experiments with blue light in mouse models often rely on implants or powerful light sources,[3b,15] so we tested if CPH1S-o can be activated directly and transdermally in tissues. Indeed, illuminating HEK293 cells transfected with rtrkB–CPH1S-o through synthetic muscle, skin, and skull (Figure S5) resulted in potent activation of signaling (Figure S6). Notably, the applied light intensities were easily attained with light-emitting diodes and were even lower than those used in photodynamic therapy.[16] For mouse abdomen (including skin, muscle, fat, and spine; thickness 10 mm; Figure S7), we found that the MAPK/ERK pathway was activated by red light in INS-1E cells transfected with mFGFR1–CPH1S-o ($I = 3.0$ mW cm^{-2}, $\lambda \approx (647 \pm 35)$ nm; Figure S8). We chose INS-1E cells, an insulinoma model for pancreatic β cells,[17] for these experiments because β-cell signaling is regulated by FGFs and because β cells are located about 10 mm under the mouse skin. Collectively, these data show the activation of cell signaling through mammalian tissues, thereby opening avenues to further non-invasive in vivo optogenetic studies.

Light-sensitive proteins that undergo reversible inter- or intramolecular binding reactions are the motors of many optogenetic methods. By identifying and adapting the sensory domain of the cyanobacterial phytochrome CPH1, we introduced an approach for the stoichiometric, red-light-induced homodimerization of proteins. Such a light-induced dimer assembly may be applied to regulate cell adhesion, gene transcription, or the cytoskeleton, potentially also by forming larger complexes after incorporation of multiple photoreceptor domains.

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